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14. ABSTRACT This proposal centers on elucidating the functional role of specific candidate genes with respect to breast carcinoma invasion. Using a short hairpin RNA interference (shRNA) approach I have previously shown that the Rho family GTPases RhoA and RhoC both contribute to an invasive phenotype. These genes have been identified as key players regulating cellular invasion. With emerging technological advances, a single candidate gene approach is now too narrow and I have taken a more global and unbiased screening approach using small interfering RNA (siRNA) molecules to identify novel genes that regulate cell motility and invasion in normal MCF10A and invasive SUM-159 breast cells. This high throughput screen will also allow dissection of the up and downstream signaling components of RhoA and RhoC. Identification of genes that have conserved functions and those that have specific motility roles will be invaluable, whilst determining those that can evoke a transition from normal to a completely motile phenotype will significantly advance our studies on breast carcinoma invasion. Likewise, finding new candidates that will inhibit motility of invasive breast carcinoma cells provides more targets of direct clinical relevance. Pathway analysis is currently underway for genes that scored in the first round of screening.					
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Introduction

This project is focused on elucidating the mechanisms involved in breast carcinoma invasion. Using a short hairpin RNA interference (shRNA) approach I have previously shown that the Rho family GTPases RhoA and RhoC both regulate invasive behaviour (1). These genes have been implicated in cell invasion within a range of tissues, with specific data emerging on melanoma and prostate carcinoma within the past year (2, 3). There is still relatively little known regarding the regulation of RhoC, although it has been suggested to function via both the MAPK and PI3K pathways (4, 2). Recently we have shown in colon carcinoma cells that RhoC expression is regulated by the Ets1 transcription factor and that increased RhoC expression correlates with a poor survival prognosis in patients with colon carcinoma (Bellovin et al., submitted). With emerging technological advances, a single candidate gene approach as outlined in my original proposal is now too narrow. As such, I have moved to take an unbiased approach to identifying key regulatory functions of both RhoA and RhoC alongside other Rho family GTPases and critical upstream and downstream players by developing a high throughput siRNA screen for cell motility. The pilot study is extremely promising and I anticipate novel insights into genes important for regulation of invasive breast carcinoma, both for initiation of the invasive, mesenchymal transition and inhibition of already invasive cells.

Body – progress report

Within Year 1 of funding I reported the development of a RhoC antibody in collaboration with Bethyl, as commercial antibodies are very poor for RhoC detection. This antibody was also weak for western blotting purposes but has proven to be highly specific when optimised for immunohistochemistry. We used this to screen a colon carcinoma tissue microarray (566 samples) and causally associated increased RhoC expression in colon carcinoma with poor prognosis and invasive carcinoma (Bellovin DI, **Simpson KJ**, Danilov T, Maynard E, Rimm DL, Oettgen P and Mercurio AM. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. Currently under revision at *Oncogene*). This data is consistent with similar findings published by Sophia Merajver's group; whilst RhoC was originally identified as over-expressed in inflammatory breast cancer (5), they have recently shown that increased RhoC expression is also associated with aggressive, invasive breast cancers of non-inflammatory origin (6).

We have also shown that knockdown of RhoA or RhoC expression using shRNA constructs (1) in a weakly motile colon carcinoma cell line recapitulated our findings in breast cells, revealing a reciprocal interaction between RhoA and RhoC activation state during the Epithelial to Mesenchymal transition (EMT) (Bellovin *et al*). Bethyl are now moving towards commercial production of the RhoC antibody.

Specific Aim 2.1 - Determine the localisation of activated RhoA and RhoC in migrating cells using FRET.

At the time of writing this proposal functional FRET biosensors for the Rho family GTPases were still in their relative infancy. Indeed while the technology is rapidly advancing and new methodology is being used to localise GTPases, it has become very clear that such experiments are not a trivial undertaking and the microscopy requirements for imaging are state of the art. This Aim was intended to be undertaken as a collaboration with the laboratory of Dr. Klaus Hahn. Over the past year Dr. Hahn moved from Scripps to the University of North Carolina and very few of his lab members moved, including Dr. Olivier Pertz, the primary postdoc I interacted with to form this collaboration. These factors together with my move to Dr. Joan Brugge's laboratory ultimately meant that we did not pursue this aim. Aside from such logistical issues, Dr. Pertz has only just published his primary manuscript measuring RhoA activity in MEFs and 293T cells (7). Clearly it took longer to achieve his research objectives than originally proposed and I would not have been in a position to utilise his technical support until this paper was published.

Interestingly, they showed that RhoA activation was differently localised depending on whether activation occurred via growth factor stimulation, random migration or wound healing.

The remainder of Aim 2 was founded on establishing FRET localisation of both RhoA and RhoC in the parental SUM-159 cells and the shRNA knockdown lines. For this reason, these objectives have not been pursued.

Specific Aim 3.1 - Establish the contribution of $\alpha 6 \beta 4$ to RhoA and RhoC activation and localisation.

Previous studies in the Mercurio laboratory showed an important role for the $\alpha 6 \beta 4$ integrin in promoting breast carcinoma progression, suggesting in Clone A colon carcinoma cells that this may occur through regulation of RhoA activation (8). Using the SUM-159 invasive breast carcinoma cell line, stable knock down of the $\alpha 6 \beta 4$ integrin using an shRNA approach was performed by Dr. Elizabeth Lipscomb (9). Invasion, measured by transwell or 3-dimensional morphology after embedding in matrigel was significantly inhibited in the absence of $\alpha 6 \beta 4$ expression and we observed limited lamellipodial ruffling upon LPA stimulation, coincident with limited migration. However, RhoGTPase pull down activity assays were performed repeatedly using these cells in the presence of different extracellular substratum and LPA stimulation and we could not establish any differential activation or expression of either RhoA or RhoC in the absence of the integrin compared with control cells. These data suggest that in the SUM-159 cells at least, the $\alpha 6 \beta 4$ integrin does not regulate Rho GTPase activity. Interestingly, it was recently shown in MDA-MB-435 cells that expression of $\alpha 6 \beta 4$ increases expression of the autocrine motility factor autotaxin, resulting in enhanced cell motility (10).

The remainder of Aim 3 focused on expanding the hypothesis that $\alpha 6 \beta 4$ regulates Rho activity. Given we are unable to establish this link we will not pursue these studies further. However, as part of the siRNA screen outlined below in New Aim 4, we have included members of the LPA receptor family and these will be assessed for their impact on invasion and motility using both the SUM-159 and MCF10A cells.

New Aim 4 – Initial pilot study to develop siRNA screens to identify genes that regulate cell motility.

Please see Appendix 1 for outline of new Aims. This has been discussed with my grant advisor Dr. Katherine Moore and a copy of this Appendix will signed by our Grants office and sent to Dr. Moore at the time of submission of this document.

With the development of emerging technologies, it is now possible to broaden our original approach of investigating two candidate genes individually, to the more global and unbiased approach of screening a number of siRNAs at once to look for comparative impacts on cell motility and invasion. High throughput screening is currently the cutting edge of RNAi biology (11) and I have been extremely fortunate to gain access to these very expensive reagents through my mentors involvement with the Cell Migration Consortium, a NIH ‘glue grant’ group of distinguished investigators that focus on a diversity of cell migration studies. To test this approach I focused initially on the Rho GTPase members, developing the assay in 96 well dish format using the functions established for RhoA and RhoC through the shRNA approach (1) and Rac1, knockdown of which significantly impairs cell migration and invasion (12). I used Dharmacon SMARTpools, an aggregation of 4 individual sequences to each particular gene to develop the assay. I have focused the screen around the classic wound healing assay for cell motility whereby cells are grown to confluence, mechanically scraped to create a wound and then left to heal, or migrate, into the open space to seal the wound. This is a form of directed migration as the wounded cells release chemotactic factors to stimulate motility (13). I have developed a high throughput wounding

assay using a robot driven pin that can reproducibly and precisely wound an entire plate in 3.5 minutes (Figure 1A).

Cell density and conditions for lipid-mediated transfection of siRNAs were established using the fore-mentioned genes alongside a fluorescent-tagged housekeeping gene to determine transfection efficiency. This was initially performed manually and I have recently developed a robotic transfection protocol using a CyBio liquid handling robot. Prior to wounding the monolayer I determine the impact of the transfection on general cell metabolism/viability using Alamar Blue reagent (BioSource), a non-toxic stain that is reduced from blue to pink by cellular secretion in the media. I have optimised the assay for both the normal mammary epithelial cell line MCF10A and for the invasive SUM-159 breast carcinoma cells. Both lines are highly amenable to the wound healing assay in the presence of growth factor stimulation and the time of wounding was established for approximately 50% closure, allowing a discernible acceleration and inhibition of motility relative to controls (Figure 1B). At the conclusion of the assay the cells are fixed and stained with rhodamine-phalloidin to observe actin stress fibers and nuclei counterstained with DAPI. Using a high throughput fixed objective microscope I have optimised the capture of each wound via a matrix of 12 individual images that are stitched together such that the entire length and breadth of the wound is imaged (Figure 1C). The amount of migration is then scored on a pixel basis using MetaExpress (Molecular Devices) and normalised to mock transfection controls. All experiments are performed in duplicate.

Knockdown of RhoA and RhoC in the MCF10A cells recapitulated results observed in the SUM-159 and colon carcinoma cells using the shRNA approach (1). Knockdown of RhoA accelerates wound healing (Figure 2A) and evokes an altered morphology reminiscent of an epithelial to mesenchymal transition (EMT; Figure 2B), the cells no longer require cell to cell contact. This phenotype was confirmed using the individual constituent single siRNA sequences for RhoA, showing 100% concordance (Figure 2C). Knockdown of RhoC likewise impairs motility. Interestingly, no other members of the Rho GTPase family promote such an acceleration of motility, with the remainder impeding or not altering migration dynamics (Figure 2A). These limited set of data show the power of screening a family of genes under all the exact same experimental conditions, providing a rapid result that is directly comparable.

On conclusion that this pilot approach was highly successful, we generated a custom library from the literature of key genes and associated family members that have a role in cell motility, invasion or metastasis, not only in breast but also in other tissues. *The Aim is to distinguish the function of different isoforms with respect to cell motility and to uncover novel genes for breast invasion.* This library totals 311 genes (4 x 96 well plates) and has been screened twice using the SMARTpool siRNA in MCF10A cells. Screening using the SUM-159 cell line is projected in the future Aims (Appendix 1). In both lines, it will be of great interest to compare genes that have conserved functions and genes that have differential functions depending on the origin of the cells. In addition to generating a numerical score to quantitate wound healing, I have also adopted a visual classification relative to the mock transfection controls. These data, together with the metabolic score provide an overall indication of the effect on cell motility. Figure 3 shows the range of phenotypes observed within the assay and Figure 4 shows a gross overview of the distribution of phenotypes for the entire library. 9.6% of the genes accelerated motility, 18% inhibited motility but were not metabolically impaired, whilst 21.2% showed impaired motility and impaired viability/metabolism. Whether this sub-group is due to true loss of viability or loss of cell adhesion will be addressed in future studies (Appendix 1).

Whilst observing a phenotypic effect provides insight into the role of a particular gene, knowing the degree of knockdown is crucial to establish whether the result can be truly attributed to that gene. Evaluating down-regulation at the protein level in such a high throughput assay is not possible due to the limited number of cells. We have entered a significant collaboration with Dharmacon RNA Technologies to establish knockdown at the mRNA level for a subset of the custom library mentioned above and for future screens planned using the human protein kinases and phosphatases. This procedure utilises bDNA (branched DNA) technology (Genospectra), a fluorescent ELISA-like high throughput method to quantitate mRNA levels without the amplification that exists by RT-PCR methods. This is an extremely expensive undertaking and one that we could not have otherwise afforded without their collaboration. Currently there are no high throughput screening publications that have rigorously validated the knockdown efficiency of all the genes screened, thus it is anticipated our future publication will have significant impact.

Key Research accomplishments

- Elucidated functional roles for Rho GTPases in cell motility of normal breast epithelial cells MCF10A
 - RhoA accelerates migration concomitant with altered ‘mesenchymal-like’ morphology
 - RhoC inhibits cell migration
- Optimised high throughput siRNA transfections for screening in 96 well format
- Developed a high throughput wound healing assay to assess cell motility
- Designed a custom siRNA library of 311 genes that encompass multiple members of gene families that are involved in cell motility, invasion and metastasis from a range of different tissue types.

Reportable outcomes

Publications

The $\alpha 6 \beta 4$ integrin maintains the survival of human breast carcinoma cells *in vivo*.

Lipscomb EA, **Simpson KJ**, Lyle SR, Ring JE, Dugan AS and Mercurio AM. *Cancer Research* 2005; 65:10970-10976.

Rabinovitz I and Simpson KJ. The actin cytoskeleton and metastasis. In “Cell Adhesion and Cytoskeletal Molecules in Metastasis”. In press: due for release at the 2006 AACR annual meeting.

Not directly related to current DOD funding.

Shackleton M, Valliot F, **Simpson KJ**, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman G and Visvader JE. Generation of a functional mammary gland from a single stem cell. *Nature* 2006; 439:84-88.

Oral presentations

Breast Program Project Grant meeting. January 11, 2006. ‘Developing an siRNA screen to identify genes that regulate breast cell motility’.

VAV Program Project Grant meeting. February 3, 2006. ‘Identification of genes regulating cell motility using a high throughput siRNA screening approach’.

Dharmacon RNA technologies. Colorado, Feb 16, 2006. ‘Developing an siRNA screen to identify genes that regulate breast cell motility’.

Harvard Medical School Department of Cell Biology retreat. March 14, 2006. ‘Factors to consider when designing siRNA screens in high throughput’.

Abstracts

Simpson KJ, Lipscomb EA, Lyle S, Brugge JS and Mercurio AM. Functional analysis of the roles of RhoA and RhoC GTPases in invasive breast carcinoma. US Army, Department of Defense, Era of Hope meeting. Philadelphia, June 2005.

Simpson KJ, Selfors LM, Bui J, Reynolds A and Brugge JS. Identification of novel genes that regulate cell motility using a high throughput siRNA screening approach. ASCB, San Francisco, December 2005.

Training

- Full time supervision of a technical assistant
- Supervision of a graduate student for a 3.5 month rotation period
- Manuscript revisions both independently and in conjunction with Dr. Brugge
- Continued involvement in organization of the Longwood area mammary gland meeting
- Attended an evening class on advanced Photoshop at the local adult education center (weekly class for 6 weeks) to enhance my ability using such a powerful image analysis package.

Current collaborations

Dr. Devin Leake and Angela Reynolds, Dharmacon RNA technologies. Quantitation of siRNA knockdown using a high throughput screening approach.

Dr. Scott Snapper at Massachusetts General Hospital Department of Medicine, Boston. Investigating the role of N-WASP in cell motility.

Dr. Alexei Degterev, Tufts University, Boston. Characterisation of small molecule inhibitors with respect to inhibition of cell motility in breast carcinoma cells.

General comments

Moving to Joan Brugge’s laboratory has significantly enhanced my research career. The laboratory and department are highly interactive. Certainly I would have been unable to initiate high throughput siRNA screening in many labs as the resources required to purchase siRNA libraries are extreme. This project has allowed me to become part of the Cell Migration Consortium, a select group of the most prominent national and international migration biologists, including Drs. Rick Horwitz, Benny Geiger, Doug Lauffenburger, Jim Parsons, Keith Burridge, Denise Montell and Jean Swarzbauer. This group meets annually and over the course of the year we engage in video conference calls to review data. Access to such a group has already significantly impacted my research.

In addition, I am now part of two large program project grant monthly meetings, both at which I have recently presented my work. One focuses on numerous aspects of breast cancer and encompasses the eminent laboratories of Drs. Robert Weinberg, David Livingston, Myles Brown, Josh LaBaer and Peter Sicinski and the second revolves around the VAV group of exchange factors and their regulation of cell motility and includes the laboratories of Drs. Fred Alt, Tom Kirchhausen and Scott Snapper.

Conclusion

Progression of Aims 2 and 3 were hampered by Dr. Klaus Hahn's re-location and technical issues. For Aim 3 to be viable, I needed to confirm my hypothesis that the integrin $\alpha 6 \beta 4$ regulated cell motility via regulation of Rho family GTPases. After numerous attempts of significant technical merit I was unable to produce data to support this hypothesis. In the meantime, I had the opportunity to develop a high throughput screening approach to identify novel genes that regulate cell motility and invasion in breast cells. This represented immeasurable promise in terms of novel gene discovery to identify genes and pathways that regulate breast cell motility and invasion. I began an initial pilot study to investigate the feasibility of this approach using the Rho GTPase family members. This exceeded my expectations for reliability and reproducibility and I progressed to develop all aspects of the screen under robotic regulation. I have just completed screening 311 genes that are implicated in cell migration, invasion and metastasis and am currently initiating the bioinformatics necessary to mine the data. Future screens are now planned.

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Appendix

Revised Statement of work for Year 3.

Supporting data

Figure 1- Proof of wound healing concept using the high throughput robotic pin head.

Figure 2- Wound healing data after Rho GTPase family pilot screen to assess motility.

Figure 3- The range of phenotypes observed in the wound healing assay after screening the 311 migration-related siRNA library

Figure 4- Collation of data after screening the 311 gens in the migration-related siRNA library.

APPENDIX 1

Dr. Kaylene Simpson
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March 27, 2006

Dr. Katherine Moore
Grant Manager
CDMRP

RE-revised Statement of Work for DOD fellowship W81XWH-04-1-0360

Dear Dr. Moore

Further to our discussion on Friday March 24, I am submitting to you a revised Statement of Work for the final year of funding of my DOD postdoctoral fellowship. This SOW has been included as an Appendix within my Annual report to be submitted March 31.

As outlined in my Annual report, I was given the fantastic opportunity to move the research focus from single gene candidates to a more global and unbiased screening approach using small interfering RNA (siRNA) molecules in high throughput. Such an approach will 1) allow identification of novel genes that regulate cell motility and invasion in breast cells and 2) dissect the up- and downstream signalling components of the Rho GTPase family, with particular reference to the RhoA and RhoC genes that I had initially focused on. siRNA screening is currently the cutting edge of mammalian siRNA technology, is still extremely expensive, requires access to robotic facilities, high throughput microscopy, extensive data storage and intensive bioinformatics analysis. Within the Department of Cell Biology at Harvard Medical School I have gained access to all the tools necessary to successfully undertake such research. These resources are certainly not available in many locations.

I therefore initiated a pilot study to investigate the potential application of this technology. The assay centers on wound healing, a well established method for measuring cell motility and was developed using the RhoA and RhoC functional phenotype previously established in the invasive SUM-159 cells and also extended to the normal breast epithelial cell line MCF10A. Such a comparison of normal versus highly invasive breast cell lines is extremely useful to identify genes that have conserved functions and those that have specific roles in regulating motility depending on the tissue of origin. To identify genes that can evoke a transition from normal to a completely motile phenotype will significantly advance our studies on breast carcinoma invasion. Likewise, finding new candidates that will inhibit motility of an already invasive breast carcinoma cell line provides more targets of direct clinical relevance.

This pilot study laid the ground work for larger scale screening. Using 96 well dishes, I developed a robotic transfection strategy coupled with a robot controlled wound healing pin that precisely and reproducibly scrapes the cell monolayer to create the wound. Imaging was performed using a high throughput fixed objective microscope and programs developed for quantitation and data normalisation. I have recently screened a library of 311 genes encompassing key genes and associated family members that have been implicated in cell migration in a range of tissues. This is the tip of the discovery iceberg and I am currently mining the data to establish regulatory networks impacted by the genes that scored as hits.

Future Aims as outlined in the revised SOW are directed towards screening the human protein kinases and phosphatases in both MCF10A and SUM-159 cells, collation of all data sets and tertiary analysis to begin to tease out pathways that are critical for breast cell migration and invasion. There is no question that this study represents a significant advance over the original proposal and stands to rapidly accelerate our understanding of the genes and signalling networks that regulate breast carcinoma invasion. Given the novelty of siRNA screening in high throughput and the direct relevance to breast cancer, I expect to publish these data by the end of the funding period in a high impact journal and anticipate involvement in projects that evolve from this screening to further delineate invasive pathways.

As ever, the continued support of the DOD is gratefully appreciated.
Yours sincerely

Kaylene Simpson

Revised Statement of work for DOD fellowship W81XWH-04-1-360 for Year 3 of funding – March 2006

Please note, a copy of this SOW document will be sent to Dr. Kathy Moore at the time of submission of the annual report.

Task 4) Develop high throughput siRNA screening technology to take an unbiased approach to identify novel genes that regulate motility and invasion of breast cells.

- 4.1 *Complete screening of the migration-related siRNA library initiated in the pilot study phase. Months 24-25*
 - Screen the SMARTpool siRNA using the invasive SUM-159 breast carcinoma cells.
 - Validate the data from the SMARTpools using 2 individual sequences to each gene in both MCF10A and SUM-159 cell lines.
 - Compare data from both lines to find genes that have conserved functions and those that are cell line specific using bioinformatics approaches. These will be further analysed in Aim 4.3.
- 4.2 *Screen the human protein kinase and phosphatase siRNA libraries using the wound healing assay approach. Months 26-27*
 - Screen the SMARTpool siRNA in both MCF10A and SUM-159 cells.
 - Assess the extent of gene knockdown after screening the SMARTpool siRNA in the MCF10A cells in collaboration with Dharmacon RNA technologies.
 - Validate the data from the SMARTpools using 2 individual sequences to each gene in both MCF10A and SUM-159 cell lines.
- 4.3 *Collate data from all screens, segregate into functional categories and analyse the data for pathway linkages using bioinformatics approaches. Months 28-29*
 - Group genes into functional classes for acceleration and inhibition of motility and those in which cell metabolism has been impaired.
 - Mine pathway analysis programs (for example, NIH DAVID) to establish relevant signalling pathways for gene hits.
 - Establish those genes with known functions in cell migration and those that represent novel findings.
- 4.4 *Develop tertiary assays to further subdivide functional categories and follow up critical candidates at a biochemical level. Months 30-33*
 - For genes that accelerate wound healing develop high throughput video microscopy using vital dye fluorescence to track cell morphology during wound closure. This should identify genes that evoke a morphological change and allow determination of migration characteristics such as directionality, persistence, velocity and lamellipodia formation.
 - Bin genes that accelerate wound healing based on expression and localisation of cell-cell adhesion markers such as E-cadherin and N-cadherin. These data will provide an

indication of whether MCF10A cells have undergone an EMT-like transition to become motile.

- Distinguish between loss of adhesion and impaired metabolism by screening this gene subset in MCF10A cells over-expressing anti-apoptotic Bcl₂.
- Chose candidate genes from these tertiary screens and from the pathway analysis in Aim 4.3 to perform large scale knockdown and biochemical validation.

4.5 *Identify genes that interact with RhoA and RhoC. Months 34-36.*

- Screen the SUM-159 RhoA and RhoC shRNA cell lines against cherry picked genes from the functional classes that accelerate and inhibit wound healing.
- Take similar bioinformatics approaches from Aim 4.3 to investigate pathways that restore or enhance the functional phenotypes of these genes.

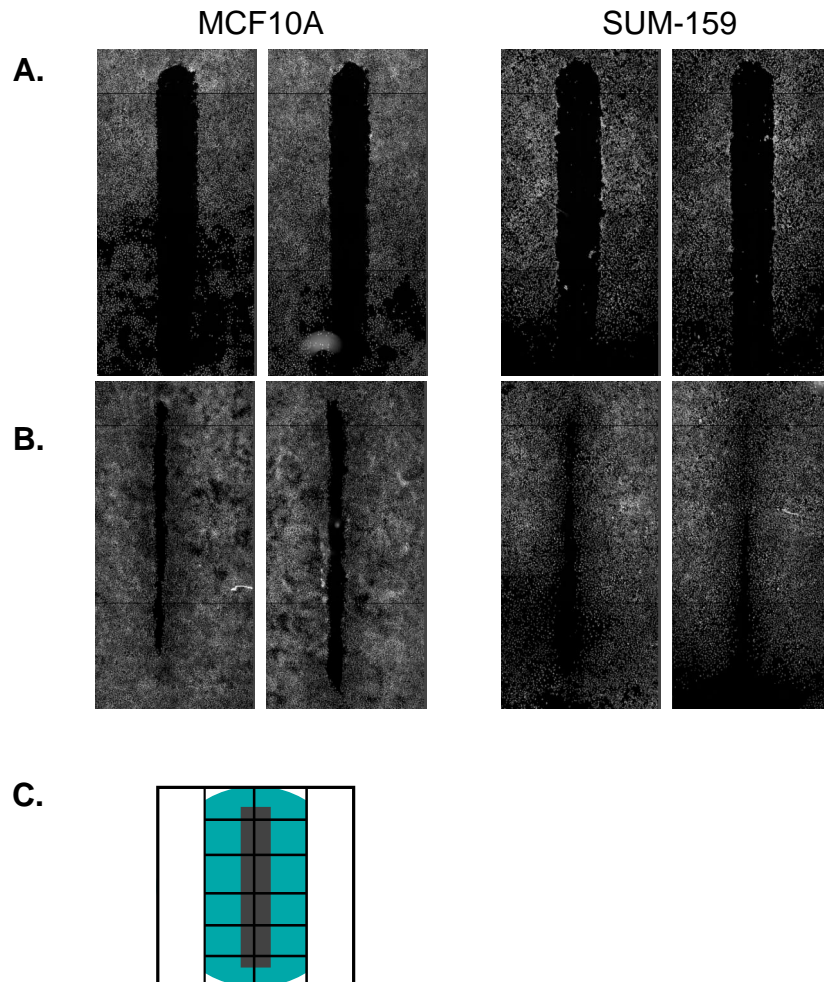


Figure 1- Proof of wound healing concept using the high throughput robotic pin head to wound a monolayer of MCF10A and SUM-159 cells. Cells were wounded then fixed and stained with phalloidin and DAPI immediately (T_0 , part A) or 12 hours post-wounding after culture in the presence of standard growth media (part B). This represents approximately 50% closure, allowing for determination of accelerated or inhibited motility. The entire wound is imaged using an Applied Precision CellWorx microscope and is the collation of 12 independent panels from a 2x6 matrix stitched together (part C).

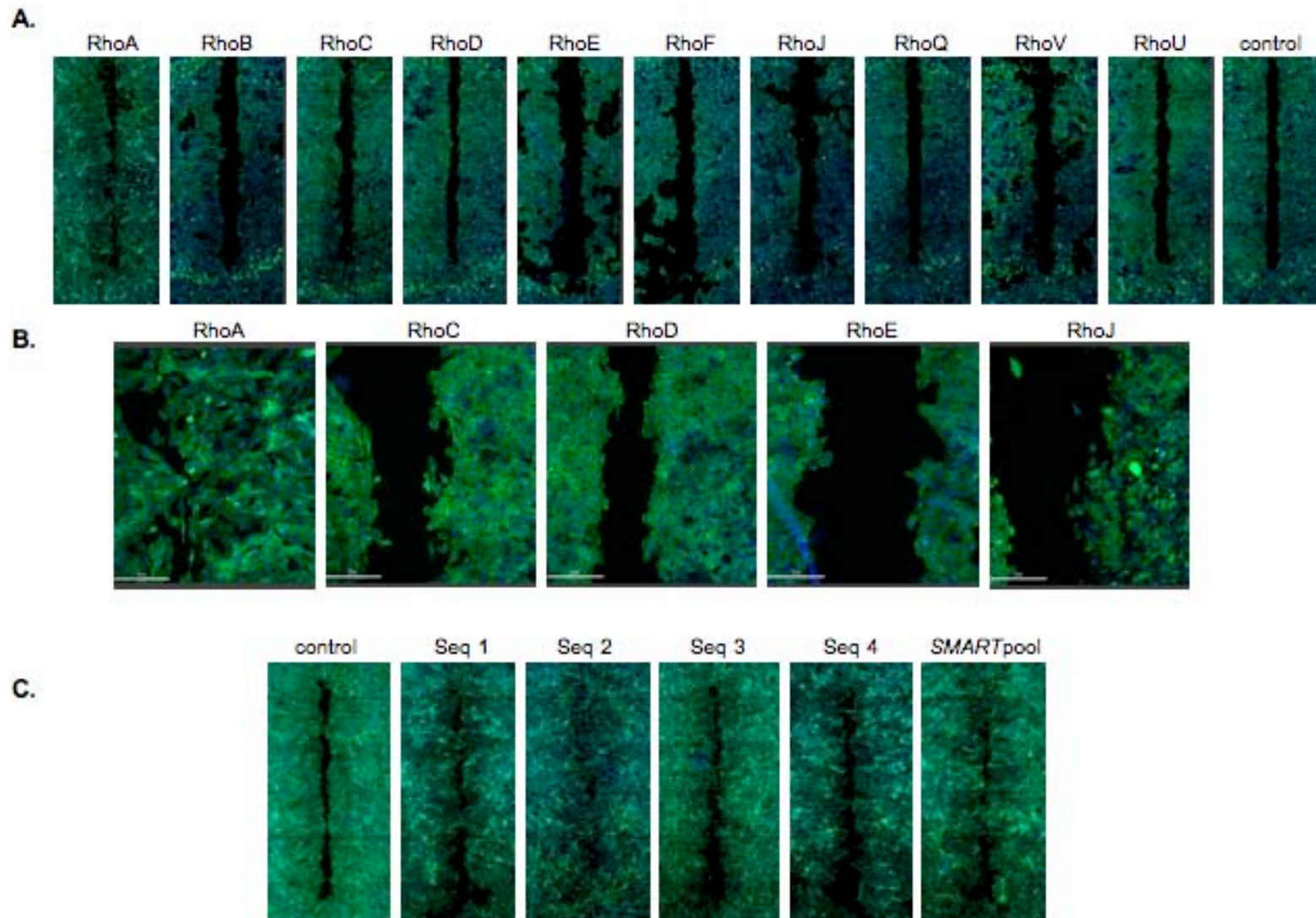


Figure 2- Wound healing data after Rho GTPase family pilot screen to assess motility. Part A) Phalloidin and DAPI stained images 12 hours post-wounding. RhoA predominantly accelerates wound healing compared to all other isoforms. Part B) higher resolution images show the altered morphology reminiscent of an EMT-like phenotype after knockdown of RhoA. Knockdown of RhoJ also evokes an interesting phenotype. Part C) Deconvolution of the RhoA SMARTpool to its individual constituent sequences. All show concordance in accelerating wound closure and the EMT morphology.

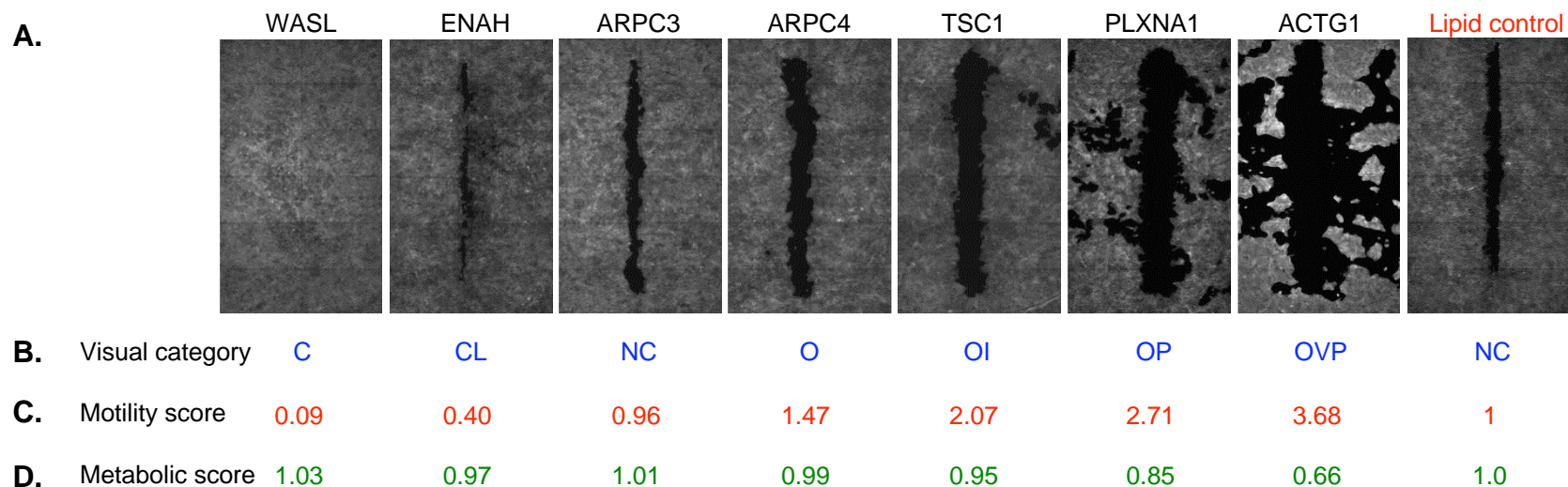


Figure 3- The range of phenotypes observed in the wound healing assay after screening the 311 migration-related siRNA library. MCF10A cells were transfected for 56 hours prior to assessment of cell metabolism/viability, followed by wounding and fixation with phalloidin and DAPI 12 hours post-wounding. Motility and metabolic scores are averaged and normalised to the mock transfection lipid controls and the visual classification is made by comparing duplicate wells relative to the lipid controls. Part A) gene names are indicated above each image and the full spectrum from closed through to very open is shown. Part B) visual classification of each wound, C=closed, CL=closing, NC=no change, O=open, OI=open with intact monolayer, OP=open with patchy monolayer; OVP= open with very patchy monolayer. Part C) the motility score is the average of duplicate wells relative to the lipid control and generally reflects the visual score. However, in instances where the monolayer may have torn during wounding, the score is then inflated and we become more reliant on the visual classification. Part D) the metabolic score correlates with loss of monolayer integrity.

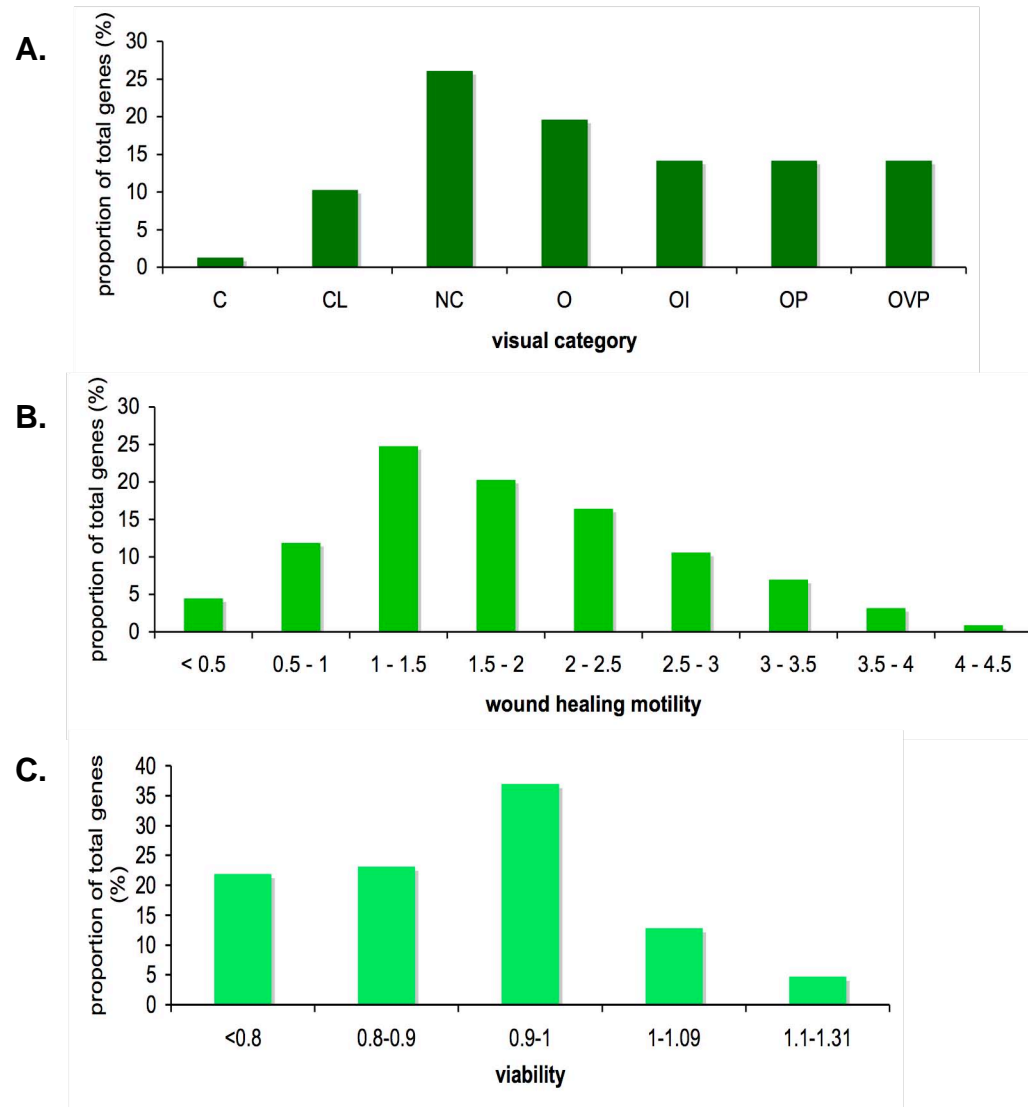


Figure 4- Collation of data after screening the 311 genes in the migration-related siRNA library. Following the criteria in Figure 3, genes were collated as a proportion of total screened and graphed according to classification. Part A) a relatively small proportion of the genes accelerated wound closure, whilst a far greater number impaired monolayer integrity. This could be due to either disruption of adhesion or loss of viability. Given the bias in this library towards migration/adhesion related genes the first is most likely the major contributor to this phenotype. Part B) numerical analysis of wound healing reflects the visual classification but shows a more normal distribution. Part C) as predicted from the visual classification, a significant number of genes showed impaired metabolism/viability.